Biochimica et Biophysica Acta, 769 (1984) 220–230
Elsevier

BBA 71935

EFFECTS OF pH ON THE INTERACTION OF LIGANDS WITH THE ($H^+ + K^+$)-ATPase PURIFIED FROM PIG GASTRIC MUCOSA

MAGNUS LJUNGSTRÖM [a], FELIX V. VEGA [b] and SVEN MÅRDH [a]

[a] *Department of Medical and Physiological Chemistry, Biomedical Centre, Uppsala University, Box 575, S-751 23 Uppsala (Sweden) and* [b] *Departamento de Biologia, Facultad de Ciencias Exactas, Naturales y Biológicas, Universidad Nacional Mar del Plata, Funes y San Lorenzo, 7600 Mar del Plata (Argentina)*

(Received July 11th, 1983)

Key words: ($H^+ + K^+$)-ATPase; Proton transport; Ligand affinity; Carbonic anhydrase; Gastric secretion

The effects of K^+, Na^+ and ATP on the gastric ($H^+ + K^+$)-ATPase were investigated at various pH. The enzyme was phosphorylated by ATP with a pseudo-first-order rate constant of 3650 min^{-1} at pH 7.4. This rate constant increased to a maximal value of about 7900 min^{-1} when pH was decreased to 6.0. Alkalinization decreased the rate constant. At pH 8.0 it was 1290 min^{-1}. Additions of 5 mM K^+ or Na^+, did not change the rate constant at acidic pH, while at neutral or alkaline pH a decrease was observed. Dephosphorylation of phosphoenzyme in lyophilized vesicles was dependent on K^+, but not on Na^+. Alkaline pH increased the rate of dephosphorylation. K^+ stimulated the ATPase and *p*-nitrophenylphosphatase activities. At high concentrations K^+ was inhibitory. Below pH 7.0 Na^+ had little or no effect on the ATPase and *p*-nitrophenylphosphatase, while at alkaline pH, Na^+ inhibited both activities. The effect of extravesicular pH on transport of H^+ was investigated. At pH 6.5 the apparent K_m for ATP was 2.7 μM and increased little when K^+ was added extravesicularly. At pH 7.5, millimolar concentrations of K^+ increased the apparent K_m for ATP. Extravesicular K^+ and Na^+ inhibited the transport of H^+.The inhibition was strongest at alkaline pH and only slight at neutral or acidic pH, suggesting a competition between the alkali metal ions and hydrogen ions at a common binding site on the cytoplasmic side of the membrane. Two H^+-producing reactions as possible candidates as physiological regulators of ($H^+ + K^+$)-ATPase were investigated. Firstly, the hydrolysis of ATP per se, and secondly, the hydration of CO_2 and the subsequent formation of H^+ and HCO_3^-. The amount of hydrogen ions formed in the ATPase reaction was highest at alkaline pH. The H^+/ATP ratio was about 1 at pH 8.0. When CO_2 was added to the reaction medium there was no change in the rate of hydrogen ion transport at pH 7.0, but at pH 8.0 the rate increased 4-times upon the addition of 0.4 mM CO_2. The results indicate a possible co-operation in the production of acid between the $H^+ + K^+$-ATPase and a carbonic anhydrase associated with the vesicular membrane.

Introduction

In the mammalian stomach, acid is produced by the parietal cells which are located to the corpus or fundus regions [1]. These cells have a characteristic morphology. Resting cells contain tubulovesicular membranes, while in secreting cells these membranes are transformed into large secretory canaliculi [2,3]. The ($H^+ + K^+$)-ATPase, which is a part of the proton translocating system, is present in the tubulovesicular membranes and in the microvilli of the secretory canaliculi [4]. Vesicles derived from the microsomal fraction of the gastric

Abbreviations: SDS, sodium dodecyl sulphate; Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; CDTA, cyclohexylene-(1,2)-dinitrilotetraacetic acid.

0005-2736/84/$03.00

mucosa contain $H^+ + K^+$-ATPase. When ATP is added to vesicles containing KCl, H^+ is transported into the vesicles in an electro-neutral exchange for intravesicular K^+ [5]. This results in an accumulation of hydrochloric acid in the vesicles. Incubation of fresh vesicles, prepared from unstimulated animals, in a medium containing KCl gives a low ATPase activity, which appears to be due to slow influx of K^+. If a K^+-ionophore, e.g., valinomycin, is added the intravesicular concentration of K^+ is no longer rate-limiting and consequently the ATPase activity increases [6]. In lyophylized vesicles the ATPase activity is high, since the permeability for K^+ is increased [7].

ATPase containing vesicle membranes exhibit a *p*-nitrophenylphosphatase activity [8]. The *p*-nitrophenylphosphatase activity is dependent on extravesicular K^+. The phosphorylation of the ATPase by ATP is inhibited by K^+ and Na^+[9,10] probably by binding to a cytosolic site [7]. The rate of dephosphorylation is stimulated by K^+ on the luminal side [11].

The cytoplasmic pH of the parietal cell depends on the secretory activity. pH increases upon acid secretion, since H^+ is withdrawn from the cytoplasm. The aim of the present investigation was to characterize the effects of K^+ and Na^+ on ATPase, *p*-nitrophenylphosphatase and hydrogen-ion transport at various pH levels. The results show that binding of alkali metal ions to a cytosolic site inhibits the enzymatic activities at alkaline pH but at a slightly acidic pH, 10–20 mM of alkali metal ions do not inhibit the $(H^+ + K^+)$-ATPase. The implication of these findings in the regulation of acid secretion is discussed.

Materials and Methods

Materials. Gastric vesicles were prepared from fresh pig stomachs as described [12]. Judged from polyacrylamide gel electrophoresis in SDS, they contain one major protein with a molecular mass of about 93 kDa, identified as the $(H^+ + K^+)$-ATPase. The vesicles were used either fresh (tight) or lyophylized (leaky). The disodium salt of ATP was purchased from Boehringer Mannheim. It was converted into its Tris salt by passage over a Dowex AG 50W-X8. Carrier-free [γ-^{32}P]ATP was purchased from New England Nuclear. The diTris salt of *p*-nitrophenyl phosphate was purchased from Sigma. Acridine orange was from Merck. All chemicals used were of analytical grade or the highest purity available.

ATPase activity. ATPase activity was assayed at 21°C as the release of $^{32}P_i$ from [γ-^{32}P]ATP [13]. The reaction medium comprised 2 mM $MgCl_2$/2 mM Tris-[γ-^{32}P]ATP. The buffers used were 30 mM Mes adjusted with Tris to pH 6.0 and pH 6.7, 30 mM Hepes adjusted with Tris to pH 6.9 and pH 7.4, and 30 mM Tris adjusted with HCl to pH 8.0. Additions of KCl and NaCl in the buffers were as indicated. The time of incubation was 10 min. The enzyme concentration was adjusted so that not more than 20% of ATP was hydrolyzed.

p-Nitrophenylphosphatase activity. p-Nitrophenylphosphatase activity was assayed at 21°C in a medium comprising 2 mM $MgCl_2$/3 mM *p*-nitrophenyl phosphate. Buffers used were 30 mM Mes adjusted with Tris to pH 5 and pH 6, 30 mM Hepes adjusted with Tris to pH 7, 30 mM Tris adjusted with HCl to pH 8 and pH 9. Reaction volume was 0.5 ml. Additions of KCl and NaCl in the buffers were as indicated. The reaction was stopped by the addition of 25 μl 50% (w/v) trichloroacetic acid. 1 ml of 0.5 M Tris-base was added and the absorbance at 410 nm was measured.

Transient-state kinetics. The technique for measuring partial reactions and calculation of rate constants was as previously described [14]. In a rapid-mixing apparatus about 0.1 mg of membrane protein was phosphorylated at 21°C for various times in the presence of 5 μM [γ-^{32}P]ATP. The reaction was stopped by expelling the reaction mixture into 5 ml 10% $HClO_4$ (w/v)/5 mM ATP/20 mM P_i. Alternatively, KCl was added in order to dephosphorylate the phosphoenzyme before stopping the reaction with acid. The acid-denatured protein was collected on a 3 μm pore size Millipore filter. The filter was washed with 7×10 ml 5% $HClO_4$ (w/v)/10 mM P_i. Then the filter was dissolved in 5 ml of acetone, and the Čerenkov radiation produced by ^{32}P was measured in a liquid scintillation counter.

In order to compare phosphoenzyme levels of different preparations of $(H^+ + K^+)$-ATPase relative units are used. One unit of phosphoenzyme, EP (relative) represents the steady-state level of

^{32}P incorporated per mg of protein at standard conditions, i.e., 21°C, 2 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP in 40 mM Tris-HCl (pH 7.4). Irrespectively of whether tight gastric vesicles or lyophilized membranes were used, the steady-state levels of phosphoenzyme were the same, indicating that the site of phosphorylation exclusively was located on the outside of the vesicles.

Transport of hydrogen ions in vesicles. Transport of hydrogen ions was measured by a spectrophotometric method as described [12,15]. Fresh vesicles were equilibrated in 150 mM KCl/2 mM $MgCl_2$ in a 10 mM buffer adjusted to the pH used in the experiment, unless otherwise is indicated. The total reaction volume was 1 ml. 10 μM acridine orange was included in the assay medium. Upon the addition of ATP the vesicles accumulated acid. When CO_2 was included in the assay, it was supplied by the addition of the indicated buffer saturated with a gas mixture containing 5% CO_2 and 95% O_2. The concentration of CO_2 in the buffer was calculated according to the Henderson-Hasselbalch equation and an appropriate aliquot was added to the incubation medium to give the final concentrations as indicated.

Protein assay. Protein was assayed according to the methods of Bradford [16] or Lowry et al. [17].

Results

Phosphorylation of ($H^+ + K^+$)-ATPase. The rate of phosphorylation was determined at pH 6.0, 7.0 and 8.0. The enzyme in a buffer of indicated pH was mixed with 2 mM $MgCl_2$ and 5 μM ATP in the presence or absence of 5 mM KCl or 5 mM NaCl (Fig. 1). Sucrose was used in order to maintain isoosmolarity and the vesicular structure. The additions of alkali metal ions results in a decrease in both rate and extent of phosphorylation at pH 7.0 and 8.0, while no effect could be observed at pH 6.0.

An enzyme-ATP complex was formed for about 120 ms by mixing enzyme with 5 μM ATP/2 mM Tris-HCl (pH 7.4). At this time 2 mM $MgCl_2$ plus buffers of various pH were added and a phosphoenzyme complex was formed. At pH 8 a pseudo-first-order rate constant of 1290 min^{-1} was observed (Fig. 2). A decrease in pH resulted in both an increased rate and extent of phosphorylation. The most rapid phosphorylation was at pH 6.0 with a pseudo-first-order rate constant of 7900 min^{-1}. Rate constants are summarized in Table I. Highest amount of phosphoenzyme was obtained at pH 7.0.

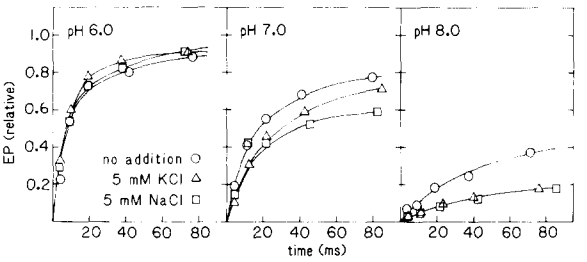


Fig. 1. Phosphorylation of ($H^+ + K^+$)-ATPase in vesicles at various pH in the presence of KCl or NaCl. Vesicles were mixed with 2 mM $MgCl_2$, sucrose to maintain isoosmolarity, and buffers of various pH. At zero time 5 μM [γ-^{32}P]ATP, 2 mM $MgCl_2$ and sucrose was added. Buffers used were 24 mM Mes-Tris (pH 6.0); 24 mM Hepes-Tris (pH 7.0), and 24 mM Tris-HCl (pH 8.0). (○——○) no additions of ions; (△——△) 5 mM KCl; (□——□) 5 mM NaCl.

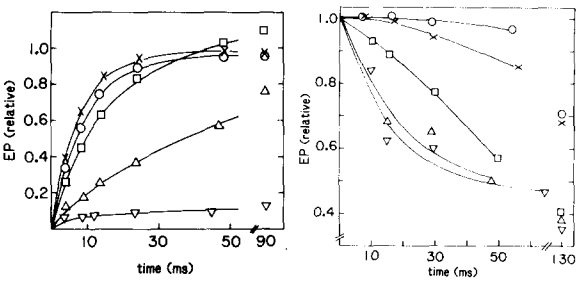


Fig. 2. Phosphorylation of ($H^+ + K^+$)-ATPase at various pH. Vesicles were mixed with 5 μM [γ-^{32}P]ATP/2 mM Tris-HCl, (pH 7.4); 0.3 mM CDTA was included in order to chelate traces of Mg^{2+}. At zero time, 2 mM $MgCl_2$ in various buffers was added. The buffers were 10 mM Hepes-Tris (pH 7) (□——□); 10 mM Mes-Tris (pH 6) (×——×); 10 mM Mes-Tris (pH 5) (○——○); 10 mM Tris-HCl (pH 8), (△——△); 10 mM Tris-HCl (pH 9) (▽——▽).

Fig. 3. Dephosphorylation of [^{32}P]phosphoenzyme at various pH. Lyophilized vesicles were phosphorylated for 120 ms in the presence of 5 μM [γ-^{32}P]ATP, 2 mM $MgCl_2$/2 mM Tris-HCl (pH 7.4). At zero time 2 mM KCl/0.5 mM unlabeled ATP was added in the presence of various buffers. The buffers were 10 mM Mes-Tris (pH 5.0) (○——○); 10 mM Mes-Tris (pH 6.0) (×——×); 10 mM Hepes-Tris (pH 7) (□——□); 10 mM Tris-HCl (pH 8) (△——△); 10 mM Tris-HCl (pH 9) (▽——▽). Unity EP corresponds to 1446 ± 140 (mean ± S.D., $n = 5$) pmol (^{32}P) incorporated/mg protein.

TABLE I

RATE CONSTANTS OF PHOSPHORYLATION OF $H^+ + K^+$-ATPase AT VARIOUS pH

Rate constants were calculated as pseudo-first-order rate constants from the experimental data in Figs. 1 and 2. The following reactions were studied in Fig. 1: E + MgATP → E-P + MgADP (reaction 1); in Fig. 2: E·ATP + Mg → E-P + MgADP (reaction 2).

pH	Rate constants of phosphorylation (per min)	
	Reaction 1	Reaction 2
5.0	–	6440
6.0	5947	7900
7.0	3548	3650
8.0	1047	1290
9.0	–	190

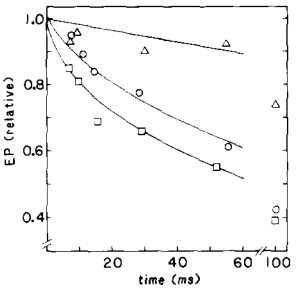


Fig. 4. Dephosphorylation of [^{32}P]phosphoenzyme by KCl and NaCl. Lyophilized vesicles were phosphorylated for 130 ms in the presence of 5 μM [γ-^{32}P]ATP/2 mM MgCl/1.0 mM Tris-HCl. At zero time, 1 mM KCl/50 mM choline chloride (○——○); 1 mM choline chlorides 50 mM NaCl, (△——△); 1 mM KCl/50 mM NaCl (□——□); was added together with 10 mM Tris-HCl (pH 8.0). Unity EP corresponds to 1127 ± 128 (mean ± S.D., $n = 3$) pmol ^{32}P incorporated/mg protein.

Dephosphorylation of ($H^+ + K^+$)-ATPase. Dephosphorylation studies were carried out on lyophilized vesicles by mixing ($H^+ + K^+$)-ATPase with 5 μM ATP/2 mM $MgCl_2$/5 mM Tris-HCl (pH 7.4). About 120 ms after this mixing, a steady-state level of phosphoenzyme was obtained. At this time, 2 mM KCl/0.5 mM unlabelled ATP was added in buffers of various pH. The unlabelled ATP was introduced in order to prevent reformation of [^{32}P]phosphoenzyme, since the velocity of this reaction was shown to vary at various pH. Highest rate of dephosphorylation was observed at alkaline pH (Fig. 3). From the initial part of the curves obtained at pH 8 and 9, a pseudo-first-order rate constant of about 2000 min^{-1} was calculated. At acid pH, there was a gradual decrease of the initial rate of dephosphorylation. At pH 5 only a very slow rate was observed. A decrease of the dephosphorylation rate by decreasing pH from alkaline to acid pH resulted in change of the shape of the curves. At alkaline pH, the curves were concave upwards, but at neutral and acidic pH they were convex. These results indicate a dephosphorylation mechanism that is different from simple first-order kinetics.

The interaction between K^+ and Na^+ on dephosphorylation was studied (Fig. 4). The phosphorylation was carried out as in the previous experiment at standard conditions at pH 7.0. After 130 ms 1 mM KCl, or 50 mM NaCl, or a mixture of both, was added simultaneously as pH was changed to 8.0. Choline-chloride was used to maintain the ionic strength. A slow rate of dephosphorylation was obtained by the addition of 50 mM Na^+ only ($k = 76$ min^{-1}). This slow rate of dephosphorylation agrees well with the ATPase activity observed in the presence of $MgCl_2$ only. The mixture of 1 mM K^+ and 50 mM Na^+ dephosphorylated the enzyme with a rate that was similar to that at 1 mM K^+ only.

Neither 10 mM KCl nor 100 mM NaCl dephosphorylated the phosphoenzyme in tight vesicles (not shown).

p-Nitrophenylphosphatase activity. Several ion transporting enzymes like ($Na^+ + K^+$)-ATPase, Ca^{2+}-ATPase as well as the ($H^+ + K^+$)-ATPase exhibit a *p*-nitrophenylphosphatase activity. The phosphatase activity of the ($H^+ + K^+$)-ATPase is dependent on K^+ or one of its congeners [18]. The reaction appears to occur on the cytoplasmic side of the membrane. In order to investigate effects of K^+ and protons on the cytoplasmic side of the ATPase, fresh gastric vesicles were used. Isoosmolarity was maintained by the addition of sucrose. The *p*-nitrophenylphosphatase activity was assayed from pH 5.0–9.0 at various concentrations of KCl (Fig. 5). In parallel experiments, 10 mM NaCl was included in the reaction medium.

The *p*-nitrophenylphosphatase activity in the absence of KCl was low and was very little af-

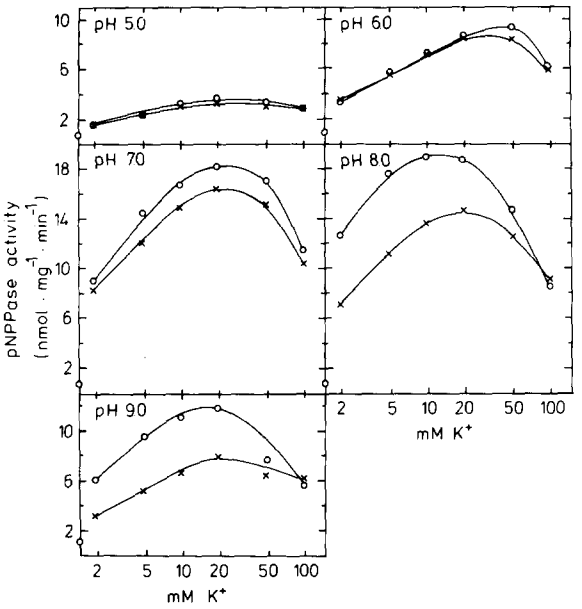


Fig. 5. Dependence of *p*-nitrophenylphosphatase activity of the $(H^+ + K^+)$-ATPase on KCl and pH. About 10 μg vesicular protein per ml were incubated for 10 min in different buffers comprising 3 mM $MgCl_2$/3 mM *p*-nitrophenylphosphate and various concentrations of KCl. The buffers were 10 mM Mes-Tris (pH 5.0 and 6.0), 10 mM Hepes-Tris (pH 7.0) and 10 mM Tris-HCl (pH 8.0 and 9.0). The experiments were performed without extra addition (○——○); or in the presence of 10 mM NaCl (×——×). Sucrose was added to maintain isoosmolarity.

fected by a change of pH. Upon an increase of the KCl concentration there was a progressive increase of the *p*-nitrophenylphosphatase activity. At pH 5.0, K^+ stimulated only slightly. The optimal activity was at pH 7.0–8.0 in the presence of 10–20 mM KCl. A further increase of the KCl concentration decreased the *p*-nitrophenylphosphatase activity. The apparent K_m was about 1–2 mM KCl at pH 7.0 and 8.0. No difference in the *p*-nitrophenylphosphatase activity was observed whether the vesicles were preincubated with KCl or not (not shown), indicating that the activation site for K^+ of the *p*-nitrophenylphosphatase was located on the cytosolic side of the vesicle.

By the inclusion of 10 mM NaCl in the reaction medium, inhibition of the *p*-nitrophenylphosphatase activity was observed. This inhibition was more pronounced at neutral or alkaline pH than at acidic pH. At the concentration of KCl giving optimal activity, 10 mM NaCl gave about 10% inhibition at pH 6.0, 11% at pH 7.0, 28% at pH 8.0 and 34% inhibition at pH 9.0. Strongest inhibition of the *p*-nitrophenylphosphatase by Na^+ was observed at low concentrations of KCl up to 10–20 mM KCl.

ATPase activity. K^+-stimulation of the $(H^+ + K^+)$-ATPase in fresh vesicles was low. In lyophilized vesicles, ions appear to have increased access to both sides of the membrane [12]. Therefore, lyophylized vesicles were used when the ATPase activity was tested at various pH and concentrations of KCl. In two parallel experiments, 10 and 25 mM NaCl was included in the reaction mixture. A progressive increase of the ATPase activity was observed upon an increase of KCl (Fig. 6). Optimal activity was observed at pH 7.4 in the presence of 10 mM KCl. The apparent K_m for KCl was decreasing from 5 mM (pH 6.0) to below 1.0 mM (pH 8.0). The ATPase activity was inhibited by high K^+ concentrations at an alkaline pH. At acidic pH, the inhibition by K^+ was less pronounced. No inhibition was observed at 100 mM KCl (pH 6.0 and 6.7). At pH 8.0, optimal activity

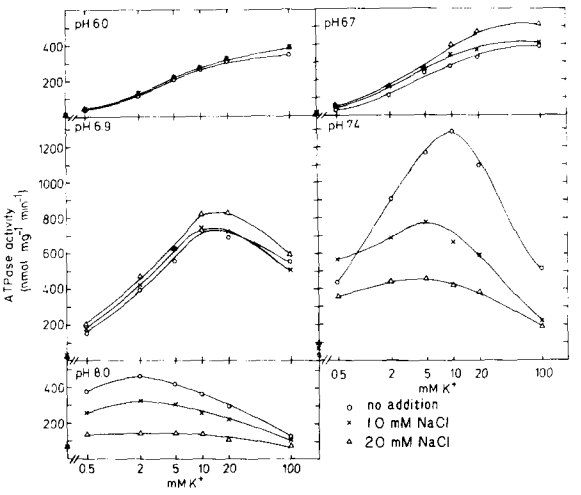


Fig. 6. Dependence of the $(H^+ + K^+)$-ATPase on KCl at various pH. Lyophilized gastric membranes, 25 μg/ml, were incubated in a medium comprising different buffers, 2 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP and various concentrations of KCl. The buffers were 4 mM Mes-Tris (pH 6.0 and 6.7), 4 mM Hepes-Tris (pH 6.9 and 7.4) and 4 mM Tris-HCl (pH 8.0). The experiments were performed in the absence of NaCl (○——○); in the presence of 10 mM NaCl (×——×) or 30 mM NaCl (△——△).

was observed at 2 mM K^+- and higher concentrations were inhibitory.

Inclusion of 10 or 25 mM NaCl, in the assay medium resulted in an inhibition of the ATPase activity at pH 7.4 and pH 8.0. At pH 6.0–6.9, no inhibition was observed. At pH 7.4 in the absence of Na^+ the ATPase activity was 1300 nmol/mg per min. When 10 or 25 mM NaCl was added the activity decreased to 57 and 37% of the maximal activity.

Effects of extravesicular cation composition on H^+ transport. Gastric vesicles were preequilibrated in 150 mM KCl (pH 7.0 or 7.5). The extravesicular concentration of Cl^- was kept constant at 150 mM, while cations were changed from either 150 mM choline or 150 mM Na^+, to 150 mM K^+. Due to dilution of the equilibrium medium, 1.35 mM K^+ was the lowest concentration of K^+ tested. Both initial rate of transport (Fig. 7A) and ΔpH (Fig. 7B) were measured. Maximal rates were obtained in a medium comprising 150 mM choline/1.35 mM K^+. The rate of H^+ transport was constant when Na^+ was exchanged for K^+. At pH 7.0, the average rate was around 0.6 of the maximal rate, while at pH 7.5 the average rate was 0.1. The ratio 10 mM K^+/140 mM choline appeared not to affect the rate at pH 7.0, but a small decrease was observed at pH 7.5. Still, at the ratio 50 mM K^+/100 mM choline at pH 7.0 no decrease was noted, while only 0.35 of the rate persisted at pH 7.5.

Maximal ΔpH registered at pH 7.0 and 7.5 were given the relative value of 1. The ΔpH did not change when choline was exchanged for K^+ at either external pH (Fig. 7B). But with an extravesicular concentration of 150–50 mM Na^+ present in the medium, ΔpH was decreased compared with the parallel experiments with choline. Increasing the concentration of K^+ increased ΔpH. Thus, it appeared as if extravesicular Na^+ produced a lower ΔpH across the membrane than did K^+, although these ions exhibited a similar ability to inhibit the rate of hydrogen ion transport.

Requirement for ATP of hydrogen ion transport. The rate of hydrogen ion transport was measured at various concentrations of ATP in vesicles preequilibrated at standard conditions. Already at micromolar concentrations of ATP there was a high rate of transport, provided the extravesicular concentration of K^+ was low (Fig. 8). The apparent K_m for ATP increased from 2.1–4.7 μM at pH 6.5 when the K^+ concentration was increased from 1.35–10 mM. This was in contrast to the great change of K_m from 7.4–37.5 μM at pH 7.5 by the same increase of K^+ (Fig. 9).

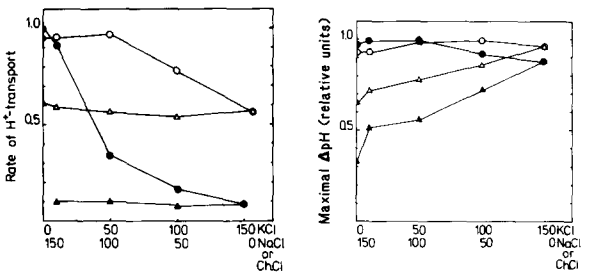


Fig. 7A and B. Dependence of the initial rate of H^+-transport and the maximal H^+-pumping capacity on the extravesicular ion composition. The vesicles were equilibrated in 150 mM KCl/2 mM $MgCl_2$/10 mM Hepes, adjusted with Tris (pH 7.0). The vesicles (12.5 μg protein) were added to a volume of 1 ml consisting of 2 mM $MgCl_2$/10 μM acridine orange/150 mM Cl^- with various ratios of Na^+ and K^+ (△——△), or with various ratios of choline and K^+ (○——○). The buffers were 10 mM Hepes adjusted to pH 7.0 or to 7.5 with Tris. Filled symbols represent results at pH 7.5 and open symbols represent results at pH 7.0. The reaction was started by the addition of 0.1 mM Tris-ATP, adjusted to the pH of the reaction medium. In A the relative rate of 1 was the extrapolated rate at 0 mM KCl and 150 mM choline. In B the maximal Δ pH at each pH was given the value of 1.

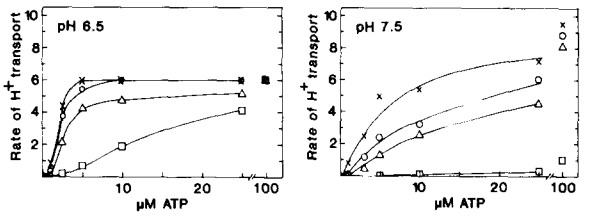


Fig. 8A and B. Dependence of the initial rate of H^+ transport in vesicles on the concentration of ATP and KCl. Vesicles were equilibrated in a medium comprising 150 mM KCl/2 mM $MgCl_2$/10 mM Hepes, adjusted to pH 7.5 with Tris. The vesicles (13 μg protein) were then transferred to 1 ml of reaction medium comprising 2 mM $MgCl_2$, 10 μM acridine orange, 10 mM Hepes, adjusted to pH 6.5 (A) or 7.5 (B) and various concentrations of KCl: 1.35 mM KCl (×——×); 5 mM KCl (○——○); 10 mM KCl (△——△); 100 mM KCl (□——□). The reaction was started by the addition of the indicated concentration of Tris-ATP, which had been adjusted to the pH of the reaction buffer.

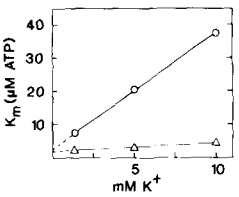


Fig. 9. Effect of extravesicular KCl on the apparent K_m for ATP. The apparent K_m for ATP was calculated from H^+-transport data shown in Fig. 8A and B. By means of a computer and the Michaelis-Menten equation, a non-linear regression analysis was performed. Apparent K_m obtained at pH 6.5 (△——△); at pH 7.5 (○——○).

Effects of extravesicular pH and CO_2 on hydrogen ion transport. The vesicles were equilibrated with 150 mM KCl. The rate of H^+ transport was measured at various concentrations of CO_2. In the absence of CO_2 the maximal transport activity was observed at pH 7.0. This activity was given the relative value of 1. The transport decreased slightly to 0.92 and 0.89 at pH 6.5 and 7.5, respectively. Only 0.18 of the original transport was observed at pH 8.0 (Fig. 10). The transport was already rapid at pH 7.0 and no change of the velocity was observed at this pH by the addition of CO_2. A slight stimulation was noticeable at pH 7.5. At 0.4 mM CO_2 the rate of transport at pH 8.0 increased to 4-times that of the control value.

Control experiments showed that the addition of CO_2 produced a very small decrease in the pH of the medium, which never exceeded 0.1 pH unit. This small decrease could not in itself explain the stimulation by the addition of CO_2 [12].

Possible formation of cytoplasmic hydrogen ions. Cytoplasmic pH is increased when the acid pump is translocating H^+ from the cytoplasm. To counteract this increase of pH, there are two processes which produce H^+. One is the formation of protons by the hydrolysis of ATP. The other is H^+ formation via hydration of metabolically produced CO_2. At pH 6.1 there is no scalar production of H^+ when ATP is hydrolysed [19]. Active uptake of H^+ in vesicles is commonly studied at this pH by means of a pH-meter. As evident from the present investigation, the $(H^+ + K^+)$-ATPase is much less inhibited by cations at low pH than at high pH. This might explain why the inhibitory effects of alkali metal ions have not been discussed until recently [9–11]. In Fig. 11, both the initial rate and the ratio of H^+-formation from the hydrolysis of 1 mM ATP are presented. The initial rate of proton production at different pH levels represents the H^+ that is liberated per unit time as a result of the hydrolysis of ATP. The ratio H^+/ATP indicates the relationship between the amount of ATP that was hydrolyzed and the total amount of protons subsequently produced by this hydrolysis. The ratio H^+/ATP increased by an increase of pH, while the rate of H^+-production was maximal around pH 7.0. The latter corresponds well with the pH optimum of the $(H^+ + K^+)$-ATPase.

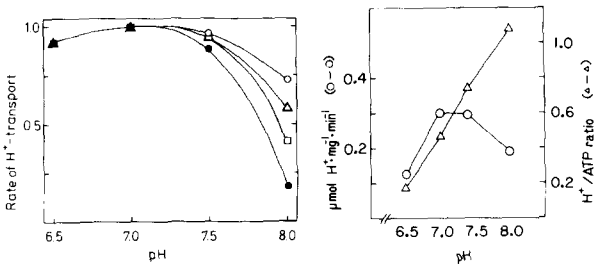


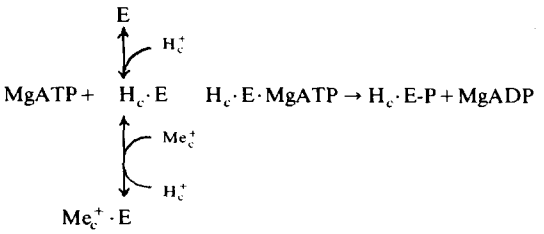
Fig. 10. Initial rate of H^+ transport at various pH and the effect of CO_2. The vesicles were equilibrated in 150 mM KCl/2 mM $MgCl_2$/10 mM buffer. The reaction volume was 1 ml and was comprised of vesicles (50 μg protein) 10 μM acridine orange, 150 mM KCl 2 mM $MgCl_2$ and various buffers: 10 mM Hepes adjusted with Tris to pH 6.5, 7.0, 7.5 and 10 mM Tris adjusted with Hepes (pH 8.0). CO_2 was supplied by the addition of the indicated buffer saturated with a gas mixture comprising 5% CO_2/95% O_2. Additions: No CO_2 (●——●) 0.1 mM CO_2 (□——□); 0.2 mM CO_2 (△——△); 0.4 mM CO_2 (○——○). The reaction was started by adding 0.1 mM ATP adjusted to the pH of the incubation medium. The initial rate of transport was plotted vs. pH. The rate at pH 7.0 was given the value of 1.

Fig. 11. Proton production by hydrolysis of ATP at constant pH. About 100 μg of lyophilized vesicles were suspended in 5 ml reaction medium, comprised of 10 mM KCl/1 mM Mg^{2+} various buffers, 0.1 mM Hepes adjusted to pH 6.5, 7.0, 7.5, with Tris and 0.1 mM Tris adjusted to pH 8.0 with Hepes, 5 μmol of Tris-ATP adjusted to the pH of the reaction medium were added. The release of protons into the medium was registered with a Metrohm titration apparatus adjusted for the pH-stat mode by the automatic addition of 1 mM KOH. The ratio of the numbers of protons released for each ATP hydrolysed and the initial rate of H^+ production are presented. Maximal ATPase activity measured as release of protons in this experiment was 753 nmol/min per mg.

Discussion

Effects of K^+ and Na^+ on the partial reactions of the $(H^+ + K^+)$-ATPase were studied at various pH by means of a rapid-mixing apparatus. In phosphorylation and *p*-nitrophenylphosphatase experiments, tight vesicles were used in order to restrict the availability of the ions for the ATPase and to simplify the interpretation of experimental data. In studies of transient states, this technique made possible the sudden addition of one or two ligands which had access only to the cytosolic side of the membrane.

An enzyme which was protonated at its cytosolic side appeared to give maximal rate of phosphorylation at pH 6.0. When pH was increased, the rate decreased. Inclusion of a low concentration of alkali metal ions did not change the rate at pH 6.0. This was in contrast to parallel experiments at pH 7.0 and 8.0, where alkali metal ions equally well inhibited the rate of phosphorylation. The results imply a site on the cytosolic side of the enzyme, which can bind monovalent cations. The transfer of the terminal phosphate group from ATP to the $(H^+ + K^+)$-ATPase may be described by the following reactions (Me^+ denotes an alkali metal ion such as K^+, Na^+, Rb^+ or the non-metal NH^{+4}; subscript c denotes cytosolic, or extravesicular side):

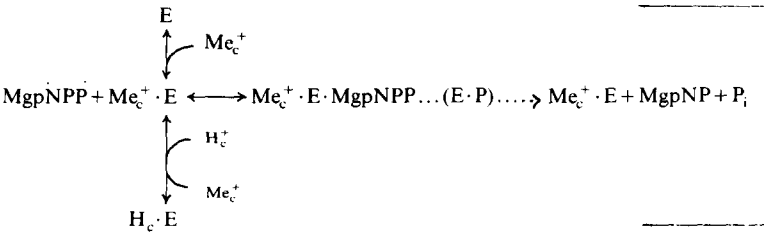


The binding of MgATP to the $H_c \cdot E$ is a fast reaction; the product $H_c \cdot E \cdot MgATP$ is unstable and is rapidly converted to the $H_c \cdot E$–P complex. The overall pseudo-first-order rate constant for these reactions is 6000 min^{-1}. This is lower compared with that of the single reaction $H_c \cdot E \cdot ATP \xrightarrow{Mg^{2+}} H_c \cdot E \cdot P$, where the pseudo-first-order rate constant is 7900 min^{-1}. Binding of MgATP to the other enzyme forms, E and $Me_c^+ \cdot E$ will result in lower rates of phosphorylation. Binding to $H_c \cdot E$ as judged from transport experiments involves a high-affinity site for ATP with an apparent K_m of 2.5 μM. This K_m was not changed in the range of pH 6.5–7.5. In the presence of K^+ on the cytosolic side, the apparent K_m for ATP increased as the concentration of K^+ was increased, indicating a decrease of the affinity for ATP. The affinity for ATP was decreased further when pH was increased in the presence of K^+, which in turn indicated that the affinity for K^+ of the unphosphorylated form of the enzyme was increased at an alkaline pH.

Dephosphorylation of the phosphoenzyme was facilitated by binding of K^+ to the luminal side of the enzyme. Na^+ in a 50-fold excess of K^+ could not inhibit the dephosphorylating ability of K^+. The results indicate that the luminal binding site for alkali metal ions has an affinity high for K^+, but very low for Na^+. Thus, this site discriminates between these two ions in contrast to the cytosolic binding site. The dephosphorylation of the $(H^+ + K^+)$-ATPase may represent a reaction sequence where protons are transported from the cytosolic to the luminal side and K^+ in the transverse direction. The dephosphorylation of the 'acid-stable' $H_c \cdot E$–P was not a simple first-order type of reaction, since the dephosphorylation curves were convex upwards and, furthermore, diphasic dephosphorylation was reported previously [11]. There are strong kinetic similarities of the dephosphorylation of the phosphoenzyme of the $(H^+ + K^+)$-ATPase and those of $(Na^+ + K^+)$-ATPase and the sarcoplasmic reticulum Ca^{2+}-ATPase. Evidence for an acid labile form of the phosphoenzyme has been reported for the two latter ATPases [21,22]. Although as yet no evidence for such a phosphoenzyme of the $H^+ + K^+$-ATPase has been presented, it is likely that it exists, taking the close similarities of the ATPases into consideration.

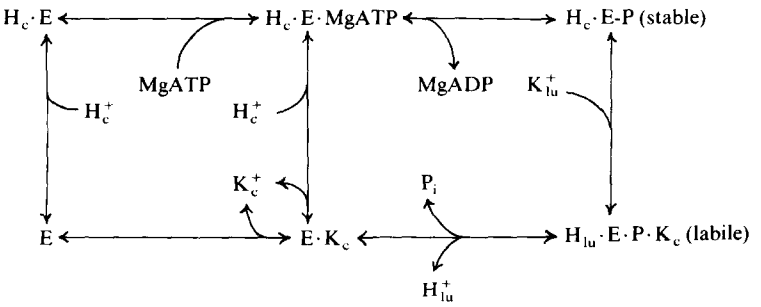
Fresh vesicles were used in the *p*-nitrophenylphosphatase experiments and changes of this activity were due to binding of ions to the cytosolic side of the vesicular membrane only. *p*-Nitrophenyl phosphate was hydrolyzed when K^+ and Mg^{2+} were present extravesicularly. The *p*-nitrophenylphosphatase activity may be described

by the following reactions:



A possible phosphorylated intermediate (E · P) may correspond to the acid labile phosphoenzyme. Me_c^+ denotes a stimulatory alkali metal ion, such as K^+, or Rb^+, or the non-metal NH_4^+ on the cytosolic side of the enzyme. Na^+ appeared to inhibit competitively this activation of the *p*-nitrophenylphosphatase. The activity of the *p*-nitrophenylphosphatase was low at acidic pH and activation required high concentrations of K^+. An extravesicular alkaline pH increased the affinity for K^+. At an alkaline pH and at moderately low concentrations of K^+, Na^+ was a good inhibitor. The extravesicular binding site appeared to have a similar pH-dependent affinity for these ions, although Na^+ was unable to catalyze the hydrolysis of the *p*-nitrophenyl phosphate.

From the present results on interactions of H^+, K^+ and Na^+ with the $(H^+ + K^+)$-ATPase in isolated tight and leaky vesicles, the following intermediary reaction sequence is summarized:



Subscript lu denotes luminal or intravesicular side. The enzyme (E) binds a cytosolic or extravesicular H^+ (H_c^+) and forms a H_c · E complex which binds MgATP at a high-affinity site. An acid-stable H_c · E-P complex is formed. Luminal K^+ binds to the phosphoenzyme which induces translocation of ions and converts the phosphoenzyme to an acid-labile form. P_i and luminal H^+ are released. The enzyme now dissociates K^+ and is rephosphorylated by ATP in a process that is accelerated by H^+ in the cytoplasm. Cytoplasmic H^+ also seems to stabilize the acid-stable phosphoenzyme by reducing the affinity for luminal K^+ (inhibits dephosphorylation). Cytoplasmic alkaline pH, on the other hand, stabilizes a K^+ form of the enzyme, which has low affinity for ATP.

The ATPase activity, measured as the release of the terminal phosphate of the ATP molecule, is a complex reaction, since lyophilized vesicles are used and alkali metal ions will bind to both sides of the enzyme. Binding of K^+ and Na^+ to the cytosolic side inhibits the ATPase activity. K^+ binds also to the luminal side and dephosphorylates the enzyme. At acidic pH, the phosphorylation is rapid and is not inhibited by alkali metal ions; the H_c · E-P form is stabilized and dephosphorylations is slow. When pH is increased, the binding of alkali metal ions to the cytosolic side inhibits the rate of phosphorylation, while the increase of pH increases the rate of dephosphorylation. Optimal conditions for the ATPase activity is obtained at pH 7.4 and 5–10 mM K^+. This concentration range of K^+ is low enough not to decrease the rate of phosphorylation, but sufficiently high to stimulate dephosphorylation. The inclusion of Na^+ decreases the ATPase activity

due to binding to the cytosolic side.

A high concentration of K^+ inhibited the *p*-nitrophenylphosphatase as well as the ATPase activity. The effect of Na^+ was additive to that of high K^+. This inhibitory action at high concentration of the alkali metal ions appears at least partly to be due to a competition between Mg^{2+} and the monovalent cation.

When extravesicular choline was exchanged for K^+, a decrease of the rate of H^+ transport was observed. This decrease was more pronounced at alkaline pH than at acidic pH. When Na^+ was exchanged for K^+ the rate of H^+ production was constant but lower than with choline chloride. The results are in good agreement with the effects of alkali metal ions on the cytoplasmic side of the membrane which inhibited the rate of phosphorylation and the *p*-nitrophenylphosphatase reaction at neutral and alkaline pH.

Regardless of the ratio of the cations, the replacement of extravesicular choline for K^+ did not affect the ΔpH. This was in contrast to the case where Na^+ replaced K^+. At high concentrations of Na^+, a lower ΔpH was obtained across the vesicular membrane. This decrease was not due to alkali metal ion inhibition on the cytosolic side as can be seen in Fig. 7A, since the replacement of Na^+ for K^+ did not change the rate of H^+ transport. The ΔpH reflects the initial concentration of K^+ inside the vesicles [12], since only K^+, not Na^+, can be exchanged for H^+. Thus the decrease of ΔpH was due to a lower intravesicular K^+ concentration. A decrease of intravesicular K^+ indicates a possible Na^+_c-K^+_{lu} exchange in the vesicular membrane.

The present investigation shows that the affinity for monovalent cations of the $(H^+ + K^+)$-ATPase is intimately linked to the pH of the surrounding medium. When the parietal cells secrete acid, this is accompanied in the cytosol by a increase of base which is transported into the extracellular fluid in the form of HCO_3^- [23]. The intracellular pH of the parietal cells appeared to be slightly alkaline with a pH around 7.4–7.8. Upon stimulation of acid secretion, the intracellular pH increased further [24,25]. There are two major processes associated with the enzyme that may take part in the physiological regulation and stimulation of the acid secretion at the level of the secretory membrane. H^+ is formed in the hydrolysis of ATP. The H^+ formed in this reaction is formed close to the H^+-binding site on the $(H^+ + K^+)$-ATPase. Secondly, in mitochondria CO_2 is produced and subsequently diffuses into the cytoplasm. Carbonic anhydrase, which is present in high concentrations in the parietal cell [26] and in the gastric vesicular membranes used in this study [12], catalyses the hydration of CO_2 to H_2CO_3, which dissociates to H^+ and HCO_3^-. The idea of cytoplasmic H^+ and CO_2 as physiological regulators of acid secretion is supported by results obtained from studies on frog gastric mucosa. An increase of CO_2 concentration in the bathing medium of the gastric mucosa increased the rate of acid secretion [27]. Another way to maintain a high rate of acid secretion in the isolated frog gastric mucosa was by lowering the pH of the serosal medium [28]. Both H^+ and CO_2 passed the basolateral membrane and decreased the intracellular pH, thus providing H^+ for the $(H^+ + K^+)$–ATPase.

The present results suggest that any reaction providing protons to the transport system on the cytoplasmic side will increase the velocity of acid accumulation into the vesicles. Furthermore, the $(H^+ + K^+)$-ATPase and carbonic anhydrase may co-operate in the secretory process. The carbonic anhydrase produces protons to 'feed' the acid pump and to counteract the alkali metal ion inhibition. Any general stimulation of the metabolism leading to the production of CO_2 and consequent formation of protons in the cytoplasm of the parietal cell, might initiate and facilitate a continuous production of gastric acid.

Acknowledgments

Thanks are due to Ms. Charlotte Carlsson for excellent technical assistance. This work was supported by the Swedish Medical Research Council, Project 13X-4965 and 4X-4965, by Uppsala University and by AB Haessle, Mölndal, Sweden.

References

1 Helander, H.F. (1981) Int. rev. Cytol. 70, 217–289
2 Ito, S. and Schofield, G.C. (1978) Acta. Physiol. Scand. Special Suppl., 25–34

3 Forte, J.G., Black, J.A., Forte, T.M., Machen, T.E. and Wolosin, J.M. (1981) Am. J. Physiol. 241, G349–G358
4 Saccomani, G., Helander, H.F., Crago, S., Chang, H.H., Dailey, D.W. and Sachs, G. (1979) J. Cell Biol. 83, 271–283
5 Sachs, G., Chang, H.H., Rabon, E., Schackmann, R., Lewin, M. and Saccomani, G. (1976) J. Biol. Chem. 251, 7690–7698
6 Forte, J.G. and Lee, H.C. (1977) Gasteroenterology 73, 921–926
7 Stewart, H.B., Wallmark, B. and Sachs, G. (1981) J. Biol. Chem. 256, 2682–2690
8 Ganser, A.I. and Forte, J.G. (1973) Biochim. Biophys. Acta 307, 169–180
9 Wallmark, B. and Mårdh, S. (1979) J. Biol. Chem. 254, 11899–11902
10 Ljungström, M., Wallmark, B. and Mårdh, S. (1979) Acta Chem. Scand. B33, 618–619
11 Wallmark, B., Stewart, H.B., Rabon, E., Saccomani, G. and Sachs, G. (1980) J. Biol. Chem. 255, 5313–5319
12 Ljungström, M., Norberg, L., Olaisson, H., Wernstedt, C., Vega, F., Arvidsson, G. and Mårdh, S. (1984) Biochim. Biophys. Acta 769, 209–219
13 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 448–463
14 Mårdh, S. and Zetterquist, Ö. (1974) Biochim. Biophys. Acta 350, 473–483
15 Lee, C.H. and Forte, J.G. (1978) Biochim. Biophys. Acta 508, 339–356
16 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 250, 7443–7449
18 Chang, H.H., Saccomani, G., Rabon, E., Schackman, R. and Sachs, G. (1977) Biochim. Biophys. Acta 465, 313–327
19 Alberty, R.A. (1969) J. Biol. Chem. 244, 3290–3302
20 Koelz, H.R., Sachs, G. and Berglindh, T. (1981) Am. J. Physiol. 241, G431–G442
21 Froehlich, J.P. and Taylor, E.W. (1975) J. Biol. Chem. 250, 2013–2021
22 Froelich, J.P., Albers, R.W., Koval, G.J., Goebel, R. and Berman, M. (1976) J. Biol. Chem. 251, 2186–2188
23 Hanke, (1931) Proc. Soc. Exp. Biol. 28, 698–700
24 Hersey, S.J. (1979) Am. J. Physiol. 237, E82–E89
25 Ekblad, E.B.M. (1980) Biochim. Biophys. Acta 632, 375–385
26 Lönnerholm, G. (1983) Acta Physiol. Scand. 117, 273–279
27 Kidder, G.W. and Montgomery, C.W. (1974) Am. J. Physiol. 227, 300–304
28 Sanders, S.S., Hayne, V.B. and Rehm, W.S. (1973) Am. J. Physiol. 225, 1311–1321